Review

Mediterranean plants as potential source of biopesticides: An overview of current research and future trends

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Review

Mediterranean Plants as Potential Source of Biopesticides: An Overview of Current Research and Future Trends

Regina Fragkouli [1], Maria Antonopoulou [2], Elias Asimakis [2], Alexandra Spyrou [2], Chariklia Kosma [1], Anastasios Zotos [2], George Tsiamis [2], Angelos Patakas [1] and Vassilios Triantafyllidis [1,*]

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Abstract: Development and implementation of safe natural alternatives to synthetic pesticides are urgent needs that will provide ecological solutions for control of plant diseases, bacteria, viruses, nematodes, pests, and weeds, to ensure economic stability of the farmers and food security as well as protection of the environment and human health. Unambiguously, production of botanical pesticides will allow sustainable and efficient use of natural resources and finally decrease the use of chemical inputs and burden. This is underlined by the strict regulations on pesticide residues in agricultural products and harmonized with the Farm to Fork strategy aimed to reduce pesticide use by 55%, by 2030. Recognizing the urgent need for natural pesticides development, this work is an overview of the current research on the valorization of Mediterranean plants as potential source of biopesticides. More specifically, the extraction methods, the chemical composition, the biopesticidal activity, the commonly used assays for evaluating the antimicrobial, the pesticidal, the repellant and the herbicidal activity of plant extracts as well as toxicological and safety aspects of biopesticides formulation are discussed in detail. Finally, the aspects that have not yet been investigated or are under-investigated and future perspectives are highlighted.

Keywords: biopesticides; plant extracts; essential oils; extraction methods; chemical composition; antimicrobial activity; insecticidal activity; herbicidal activity; alternative agriculture

1. Introduction

Climate change and environmental degradation are severe threats worldwide and their consequences can cause serious impacts on our planet. Recognizing the importance of these threats to humanity, on December 11, 2019, the EU Commission presented the European Green Deal which is consisted of a set of policy initiatives that aimed to neutralize climate by 2030 and render Europe the first climate-neutral continent by 2050 [1]. One of these initiatives is the reduction of greenhouse gas emissions by at least **55% by 2030,** compared to 1990 levels. To achieve 2030 climate targets, the EU Commission has also adopted a set of strategies in various sectors such as transportation, industry, energy, and agriculture [2].

Amongst them, the Farm to Fork strategy is characterized as the heart of the European Green Deal and aims to accelerate the transition to a sustainable food system. The objective of this Farm to Fork Strategy is to ensure food safety in an environmentally sustainable manner maximizing simultaneously environmental, health and social benefits. To accelerate the transition to sustainable and healthy food systems, this strategy aims to reduce pesticide use by 50%, by 2030, applying low-input sustainable agriculture or simply alternative agriculture, amongst others [2].

Pesticides are any substance or mixture of substances of chemical or biological ingredients intended for repelling, destroying, or controlling any pest, or for regulating plant growth [3].–The

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term pesticide applies to insecticides, herbicides, fungicides, rodenticides, molluscicides, wood preservatives and various other substances used to control pests. Pesticides also include plant growth regulators, defoliants, and desiccants. Their use has increased 50% since 1950, and it is estimated that 2.5 million tons of industrial pesticides are now used each year [4]. Moreover, global pesticide use shows an increasing trend in the future, and it is expected to reach the value of 4.5 million tons in 2030 [5,6].

Although pesticides have a principal role in crop production, their intensive and improper use can cause numerous detrimental effects on human health, the environment, and the safety of agricultural products raising major public and scientific concern in the last decades [7,8,9]. For humans, dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive, and endocrine effects are representative adverse health effects that have been associated with pesticides [10].

Considering that chemical pesticides can pose human and environmental health risks as well as the aims set by the Farm to Fork strategy, there is an increasing demand for the development of alternative eco-friendly pesticide formulations. Biopesticides have long been recognized as attractive alternatives to synthetic chemical pesticides for pest control because they present significant properties with their non-toxic nature to be the most important [11,12,13].

Biopesticide is a term that includes many aspects of pest control such as microbial (viral, bacterial, and fungal) organisms, entomophagous nematodes, plant-derived pesticides (botanicals), secondary metabolites from microorganisms (antibiotics), insect pheromones applied for mating disruption, monitoring or lure-and-kill strategies and genes used to transform crops to express resistance to insect, fungal and viral attacks or to render them tolerant of herbicide application. They are categorized into three groups: (i) microbial biopesticides (contain microorganisms like bacteria, fungi, viruses and protozoan which attack specific pest species, or entomopathogenic nematodes as active ingredients), (ii) biochemical biopesticides (based on naturally occurring substances that control pests by non-toxic mechanisms, in contrast to chemical pesticides that contain synthetic molecules that directly kill the pest) and (iii) plant-incorporated protectants (pesticidal substances that plants produce from genetic material that has been added to the plant) [11,12].

The practice of using plant derivatives in agriculture has a long history, at least two and a half millennia, in ancient Greece and Rome [14]. Botanical pesticides are characterized by bioactive mixtures/extracts/compounds from plant materials, which serve as insecticides and repellents but also as bactericides, fungicides, herbicides and nematicides [15]. Nowadays, botanical pesticides and biopesticides generally gain more and more scientific and agricultural market interest, as a non-toxic and environmentally friendly solution due to their natural composition.

As plant-based natural pesticides have gained considerable attention the last years, there is an urgent need to compile the current scientific knowledge about plants presenting biopesticidal effects especially for the countries, where the source plants are readily available and conventional formulations comprising of synthetic pesticides are both expensive and dangerous to humans and environment. Being aware of the above, numerous research articles are focused on the evaluation of extracts and essential oils with biopesticidal properties from plants of Mediterranean countries. Therefore, this study provides an overview of the current research on botanical pesticides native to Mediterranean countries for the period 2017- February 2023. The data was collected from the Elsevier-Scopus Database by performing document searches within article titles, abstracts, and keywords, using the following search criteria: the keyword “biopesticides”, the publication year: 2017-2023 (February), and the document type “article”. The generated literature list was checked manually and only the articles focused on the targeted topic were considered.

Based on the overview, the extraction methods, the chemical composition, the biopesticide activity as well as the commonly used assays for evaluating the antimicrobial, the pesticidal, the repellant and the herbicidal activity of plant extracts are discussed. Special attention is also devoted to toxicological and safety aspects that should be considered before the commercialization of

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biopesticides formulation. Finally, the gaps in the literature that should be investigated, and future perspectives are highlighted.

2. Mediterranean Plants That Have Been Recently Investigated for Biopesticidal Activity

The literature review between 2017- 2023 (February) revealed 40 families with at least one species having scientific interest as potential biopesticide. Among them, six families had the highest number of species and are presented in detail below. However, it is worth mentioning the existence of other families like Meliaceae or Rutaceae with great scientific interest, but no related articles in the examined period or species native to Mediterranean countries, that are not presented in this review paper. The biological activity of the species (and generally of the families) is determined by the chemical composition of the secondary metabolites. According to Pichersky and Gang [16], secondary metabolites are compounds whose biosynthesis is restricted to selected plant groups and serve specific needs of the plant (e.g. insect attraction, resistance to salt or drought).

2.1. Lamiaceae

The Lamiaceae (or Labiatae) is a family of plants composed of 7530 species [17] (trees, shrubs, subshrubs, and herbs), which are characterized by annual or perennial carriage [18,19]. It can be found all over the planet and has several species of aromatic plants which are used in medicine, the pharmaceutical and food industries [20] and as ornamental plants. The most interesting species with several biological applications belong to the genera *Thymus* (e.g., *Thymus vulgaris*), *Origanum* (e.g., *Origanum vulgare*), *Salvia* (e.g., *Salvia rosmarinus*) and the common garden sage (e.g., *Salvia officinalis*), *Levandula* (e.g., *Lavandula angustifolia*), *Mentha* (e.g., *Mentha spicata*), *Ocimum* (e.g., *Ocimum basilicum*) and species belonging to the genus *Melissa* [21]. Essential oils of these species have been reported to possess strong insecticidal, acaricidal, fungicidal and herbicidal activity apart from other biological activities such as antioxidant, antitumor, anti-inflammatory, antiviral, analgesic, antitussive, antiasthmatic, and antimicrobial activities [22,23,24].

All these activities are determined by the chemical composition of the essential oils. In general, the species of Lamiaceae produce large amounts of secondary metabolites and can be distinguished into two groups according to the kind of secondary metabolites:

- Those species that mainly produce volatile terpenoids in their essential oils and
- Those species that mainly produce nonvolatile metabolites and poor essential oils [19].

According to Table 1 the Lamiaceae species are rich especially in monoterpenes and sesquiterpenes as they were found to be frequent constituents of Lamiaceae essential oil. More specifically, the essential oils are characterized by large quantities of some well-known compounds like carvacrol (*Origanum*, *Satureja*, and *Thymus* species), camphor (*Lavandula* species and *S. rosmarinus*), menthol (*Mentha* species), thymol (*Origanum* and *Thymus* species) etc. which can present biological activities individually or synergistically with other compounds [25,26]. In general, the chemical composition of essential oils is affected by several factors such as species, seasonality, plant age, and geographic location as well as extraction methods [27]. For example, the composition of the essential oil of *Thymus vulgaris* varies qualitatively and quantitively among the experiments of Valcárcel et al. [28], Sarić-Krsmanović et al. [29] and Ben Jabeur et al. [30] who collected plants from different geographical locations (Spain, Serbia, and Tunisia respectively).

2.2. Asteraceae

The Asteraceae (or Compositae) is the largest family of plants in the Angiosperms [31]. It is represented by more than 24.000 described species which constitute 10% of all flowering species [32] and are characterized by annual or perennial carriage. Most of the species are herbaceous and only a small number are shrubs and trees [33]. It includes crops with nutritional (lettuce, artichoke, chicory), medicinal (echinacea and chamomile) and ornamental value (chrysanthemum, dahlia, zinnia, gerbera, and others). The family is distributed all over the world, except Antarctica [34]. The species

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of Asteraceae family have pharmaceutical applications as they possess antioxidant, anti-inflammatory, antimicrobial, diuretic, and wound healing properties) [35]. In addition, insecticidal [36] and fungicidal activity [37] has also been reported for their essential oil. The above activities are attributed to their phytochemical profile, which consists of terpenoids, lignans, saponins, polyphenolic compounds, phenolic acids, sterols, and polysaccharides [38]. The terpenoids and especially monoterpenes and sesquiterpenes are abundant [39]. The monoterpenes can act as AChE inhibitors in various insects [40], while sesquiterpenes lactones are constituents with a great biological value [41].

2.3. Apiaceae

The Apiaceae (or Umbelliferae) is a family of mostly aromatic annual, biennial, or perennial herbs and less often shrubs or trees. It consists of 442 genera and 3575 species and has a worldwide distribution mostly in the northern temperate regions and high altitudes in the tropics [42]. The family includes crops with nutritional, medicinal, and industrial use. They also can be used as beverages, spices, cosmetics, and fragrances [43]. The essential oils of a large number of species have been exploited successfully for insecticidal activity [44], fungicidal [45] and herbicidal activity [46]. These activities are correlated with their chemical composition consisting of more than 760 different constituents [47,48]. Monoterpenes, phthalides, terpenoids, phenylpropanoids (coumarins and phenylpropenes), and polyacetylenes are commonly found in Apiaceae plants [49].

2.4. Cistaceae

The Cistaceae family consists of 8 genera and 180 species (shrubs and herbs), distributed in temperate and subtropical regions of the northern hemisphere, especially the western Mediterranean region [50]. In particular, five of the eight genera (*Cistus*, *Fumana*, *Halimium*, *Helianthemum*, *Tuberaria*) are native to this region while the remaining three (*Crocanthemum*, *Hudsonia*, *Lechea*) are native to temperate regions in America [51]. The phytochemical profile of *Cistus* species and especially the high amounts of polyphenolic compounds (especially catechins) provide them with the ability to withstand extreme conditions [52]. The Cistaceae family also has a long history in medicine due to their pharmaceutical value (anti-inflammatory, antiulcerogenic, wound healing, and antimicrobial properties). The main compounds of *Cistus* essential oil are monoterpenes (pinene, borneol, camphor, and carvacrol), sesquiterpenes (viridiflorol and zingiberene) and diterpenes (manoyl oxide and abietatriene) [53]. Species of the family have been examined successfully against the fungus *Geotrichum citri-aurantii* in citrus [54].

2.5. Cupressaceae

The Cupressaceae family is a family of resinous, monoecious, or dioecious shrubs or trees (125 species) with a worldwide distribution [55]. The species present anti-inflammatory, anticancer, antimicrobial, insecticidal and antifungal activity [24,56]. They mainly contain terpenes (monoterpenes and sesquiterpenes), alkaloids (piperidines) and polyphenols (phenolic acids, flavonoids, proanthocyanidins, lignans, acetophenones, and stilbenes). The species have an important role in drug development and their phytochemicals can be used as a natural source for future drugs [57]. They also present significant repellent and insecticidal activity against various pests [58,59] and pathogens [60]. Juniper essential oils also showed promising results in weed control [61].

2.6. Brassicacae

The Brassicaceae family includes many economically important species which are cultivated for human food, animal feed, edible oil, and biofuel. A great number of weeds also belong to this family [62]. It is consisted of 3709 species and has a worldwide distribution except Antarctica [63]. The family species contain a variety of secondary metabolites and the organosulphur compounds

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(glucosinolates), the phenolic acids and the flavonoids are the most important [64]. Especially glucosinates give benefits in human health by reducing risk for degenerative diseases, but also in plant health by inducing their resistance to insects and pathogens [65]. Morra et al. [66] and Konecka et al. [67] demonstrated the herbicidal and insecticidal activity of seed meal and oil respectively from *Sinapis alba* L.

Table 1. Overview of extraction methods and isolated compounds of Mediterranean plant species.

Family/ Plant Species	Extract methods*	Major isolated compounds	references
Acanthaceae			
Acanthus dioscoridis L.	m		[44]
Amaranthaceae			
Achyranthes aspera L.	se	flavonoids, saponins, tannins, steroids, cardiac glycosides, alkaloids, anthrequinones and terpenoids	[68]
Anacardiaceae	se		
Pistacia atlantica Desf.	h	*EO leaves*: terpinen-4-ol; (p)-cymene; α-pinene; spathulenol *EO fruits*: Terpinen-4-ol; sabinene; α-pinene. *EO bark:* α-pinene; myrtenol; verbenol⟨trans->; β-pinene	[69]
Pistacia atlantica Desf.	h		[70]
Pistacia khinjuk Stocks.	h	*Fruit oil:* b-pinene; sabinene; *Leaf oil:* spathulenol; b-pinene	[70]
Pistacia lentiscus L.	se		[71]
Apiaceae			
Anethum graveolens L.	h	L-phellandrene; Carvone; limonene	[72]
Bifora radians M. Bieb.	m		[44]
Carum carvi L.	h	carvone; D-limonene; α-myrcene; dihydrocarvone	[73]
Carum carvi L.	p	limonene; carvone	[46]
Carum carvi L.	m, sub	(+) carvone; d-limonene	[45]
Coriandrum sativum L.	m		
Crithmum maritimum L.	h	dill apiole; γ-terpinene; and carvacrol methyl ether	[74]
Crithmum maritimum L.	h	Dillapiole; γ-terpinene. (French EO), limonene; γ-terpinene (central Italy EO); thymol methyl ether; γ-terpinene (Sicilian EO).	[75]
Cuminum cyminum L.	h	α-pinene; o-cymene; cuminaldehyde; ç-terpinene	[73]
Cuminum cyminum L.	p	cuminic acid	[76]
Daucus carota L.	h	α-pinene; β-pinene; borneol; myrcene	[77]

 doi:10.20944/preprints202308.0422.v1

Daucus lopadusanus Tineo	m		[78]
Foeniculum vulgare Mill.	h	anethole	[79]
Foeniculum vulgare Mill.	h	α-pinene; anethole; D-limonene; L-fenchone	[73]
Foeniculum vulgare Mill.	p	trans-anethole; limonene; fenchone	[80]
Helosciadium nodiflorum (L.) W.D.J. Koch	h	myristicin; (Z)-β-ocimene	[81]
Heracleum sphondylium L.	h	octyl acetate; octyl butyrate; octyl hexanoate	[74]
Pimpinella anisum L.	h	anethole; D-limonene; estragole; o-cymene	[73]
Pimpinella anisum L.	p	transanethole	[80]
Pimpinella anisum L.	h	(E)-anethole; Methyl chavicol	[74]
Smyrnium olusatrum L.	h	curzerene; iso-furanodiene; furanoeremophil-1-one; germacrone; myrcene	[81]
Apocynaceae			
Calotropis procera (Aiton) W.T.Aiton	se		[82]
Nerium oleander L.	m		[83]
Nerium oleander L.	se		[83]
Asclepiadaceae			
Periploca angustifolia Labill.	m		[78]
Asphodelaceae			
Asphodelus ramosus L. subsp. *ramosus*	m, ultra		[58]
Asteraceae			[71]
Achillea millefolium L.	h	chamazulene; 1,8-cineole	[36]
Achillea millefolium L.	m		[44]
Achillea millefolium L.	m, sub		[45]
Achillea ptarmica L.	m		[84]
Achillea millefolium L.	m		[84]
Anthemis deserti Boiss.	m		[85]
Arctium lappa L.	m		[84]
Artemisia inculta Delile	h	camphor (19), 1,8-cineole (12), p-cymene p, borneol	[28]
Artemisia absinthium L.	h	sabinene (23.8%) and β-myrcene (15.5%)	[36]
Bidens tripartite L.	m		[84]
Carduus acanthoides L.	m		[84]
Carduus nutans subsp. *leiophyllus* (Petrović) Stoj. & Stef.	m		[84]
Centaurea cyanus L.	m		[84]
Centaurea jacea L.	m		[84]

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Centaurea scabiosa	m		[84]
Cirsium arvense (L.) Scop.	m		[84]
Cynara cardunculus L. var. *altilis* DC.	m	caffeoylquinic acids, apigenin, luteolins, lactone cynaropicrin	[86]
Dittrichia viscosa (L.) Greuter	m	α-costic acid and inuloxin A	[87]
Dittrichia viscosa (L.) Greuter	-	α-costic acid, inuloxin A, inuloxin C	[88]
Echinops ritro L. var. *tenuifolius*	m		[84]
Echinops spinosissimus Turra	m		[78]
Gnaphalium uliginosum L.	m		[84]
Glebionis coronaria (L.) Spach	se	camphor	[89]
Leontodon hispidus L.	m		[84]
Pentanema britannica (L.) D.Gut.Larr., Santos-Vicente, Anderb., E.Rico & M.M.Mart.Ort.	m		[84]
Pulicaria crispa (Forssk.) Oliv.	m		[90]
Santolina chamaecyparissus L.	h	artemisia ketone; β-phellandrene; vulgarone B; β-myrcene	[36]
Santolina chamaecyparissus L.	h	1,8-cineole; 8-methylene-3-oxatricyclo[5.2.0.02,4]nonane	[91]
Silybum marianum (L.) Gaertn.	m		[84]
Sonchus arvensis L.	m		[84]
Tanacetum vulgare L.	m		[92]
Tanacetum vulgare L.	h	α-thujone; 1,8-cineole	[36]
Taraxacum officinale F.H. Wigg. subsp. *officinale*	m, sub		[45]
Tripleurospermum inodorum (L.) Sch. Bip.	m		[84]
Solidago virgaurea L.	h	pentadecanol; germacrene D	[29]
Boraginaceae			
Glandora prostrata (Loisel.) D.C.Thomas	se		[93]
Onosma visianii Clementi	se	isobutylshikonin; isovalerylshikonin	[94]
Brassicaceae			
Brassica rapa L.	se		[71]
Diplotaxis erucoides (L.) DC.	se		[71]
Diplotaxis virgata (Cav.) DC.	se		[71]
Hirschfeldia incana (L.) Lagr.-Foss.	se		[71]
Sinapis alba L.	m		[66]
Cannabaceae			
Humulus lupulus L.	m, sub		[45]
Humulus lupulus L.	m	α-Humulene; myrcene; trans-caryophyllene	[95]

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Caryophyllaceae			
Saponaria officinalis L.	m		[75]
Chenopodiaceae			
Atriplex halimus L.	m		[78]
Chenopodium murale (L.) S. Fuentes & al.	se	flavonoids, saponins, tannins, steroids, cardiac glycosides, alkaloids, anthrequinones and terpenoids	[68]
Cistaceae			
Cistus albidus L.	se		[71]
Cistus albidus L.	m		[54]
Cistus criticus L.	m		[54]
Cistus crispus L.	m		[54]
Cistus ladanifer L.	se		[71]
Cistus ladanifer L.	m		[54]
Cistus laurifolius L.	se		[71]
Cistus laurifolius L.	m		[54]
Cistus monspeliensis L.	m		[54]
Cistus populifolius L.	m		[54]
Cistus salviifolius L.	m		[54]
Convolvulaceae			
Convolvulus arvensis L.	se	flavonoids, saponins, tannins, steroids, cardiac glycosides, alkaloids, anthrequinones and terpenoids	[68]
Cupressaceae			
Juniperus communis L.	h	α-pinene; sabinene; β-myrcene; limonene; terpinen-4-ol; germacrene D; δ-cadinene	[59]
Juniperus communis L.	p	α-pinene; myrcene	[60]
Juniperus communis L.	-	α-pinene; sabinene; limonene	[96]
Juniperus communis var. *saxatilis* Pall.	h	α-pinene; sabinene; b-pinene; terpinen-4-ol; β-elemene	[59]
Juniperus excelsa M. Bieb.	h	α-cedrol; α-limonene; α-pinene	[61]
Juniperus oxycedrus L.	h	α-pinene; limonene; β-caryophyllene	[59]
Juniperus phoenicea L.	m, ultra		[58]
Juniperus sabina L.	h	sabinene	[61]
Dennstaedtiaceae			
Pteridium αquilinum (L.) Kuhn	m	linolenic acid; phytol; palmitic acid; stearic acid; citronellol	[97]
Eqoisetaceae			
Equisetum αrvense L.	m, sub		[45]
Fabaceae			
Cassia senna L.	m		[85]

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Retama raetam (Forssk.) Webb	m	alpinumisoflavone; hydroxyalpinumisoflavone; laburnetin; licoflavone C; retamasin B; ephedroidin	[98]
Sophora alopecuroides L.	m	alcaloids	[99]
Ulex europaeus L.	se		[93]
Hypericaceae			
Hypericum aegypticum L.	m		[78]
Hypericum perforatum L.	m, sub		[45]
Juncaceae			
Juncus compressus Jacq.	p	effusol; juncusol	[100]
Lamiaceae			
Calamintha menthifolia Host	m	gallic acid; caffeic acid; 2-hidroxy-cinnamic acid; kaempferol; callistephin chloride; p-coumaric acid; idaenin chloride; (+)-Catechin hydrate	[101]
Hyssopus officinalis L.	h	cis-pinocamphone; b-phellandrene; b-pinene	[60]
Hyssopus officinalis L.	h	1,8-cineole; b-pinene	[91]
Lavandula × *intermedia* Emeric ex Loisel.	h	linalyl acetate; linalool	[91]
Lavandula angustifolia Mill.	h	linalyl acetate; linalool; geranyl acetate; terpineol	[28]
Lavandula angustifolia Mill.	h	linalool, coumarin, α-terpineol, caryophyllene oxide and coumarin	[102]
Lavandula angustifolia Mill.	m, sub		[45]
Lavandula dentata L.	h	eucalyptol; fenchone; camphor	[103]
Lavandula angustifolia Mill.	-	β-phellandrene; 1,8-cineole; terpinen-4-ol; caryophyllene	[96]
Lavandula canariensis Mill.	m		[104]
Melissa officinalis L.	h	geranial; neral; citronellal	[29]
Mentha ×*piperita* L.	m		[105]
Mentha ×*piperita* L.	h	menthone; menthol; limonene	[28]
Mentha ×*piperita* L.	h	menthol; menthone	[46]
Mentha ×*piperita* L.	m, sub		[45]
Mentha ×*piperita* L.	-	menthofuran; menthol	[96]
Mentha spicata L.	h	carvone; 1,8-cineole; menthol	[28]
Mentha spicata L.	m		[106]

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Mentha suaveolens Ehrh.	h	piperitenone oxide; bornel	[69]
Mentha suaveolens Ehrh.	h	piperitenone oxide; piperitenone; limonene; D-germacrone; t-caryophyllene	[28]
Mentha suaveolens Ehrh.	m, ultra		[58]
Mentha x verticillata L.	se		[71]
Mentha viridis (L.) L.	m		[85]
Nepeta cataria L.	h		[107]
Nepeta curviflora Webb & Berthel.	h	2-isopropyl-5-methyl-3-cyclohexen-1-one; (-)-spathulenol; cis-Z-α-bisabolene epoxide; widdrol; (E,Z)-5,7-dodecadiene; dihydronepetalactone; and 4-propyl-cyclohexene	[108]
Nepeta nuda L. ssp. *pubescens*	h	pinene; 1-ethyl-1H-pyrrole; 1-cycloethyl-1-(2-methylenecyclohexyl)ethanol; 3-methyl-2-cyclohexen-1-one; 2,3-dimethyl-3-hexanol	[108]
Origanum elongatum (Bonnet) Emb. & Maire	h	carvacrol; p-cymene; g-terpinene	[109]
Origanum majorana L.	h		[107]
Origanum syriacum L. subsp. *syriacum*	h	carvacrol	[25]
Origanum virens Hoffmanns. & Link	h	p-Cymene; carvacrol; linalool; a-terpinene; myrcene; b-caryophyllene	[28]
Origanum vulgare L.	h		[107]
Origanum vulgare L.	h	terpinene; cis-p-menth-2-en-1-ol; terpinen-4-ol; thymol; α-terpinene	[110]
Origanum vulgare L.	se		[71]
Phlomis tuberosa L.	m		[44]
Prasium majus L.	m		[78]
Rosmarinus officinalis L.	h	verbenone, a-pinene	[111]
Rosmarinus officinalis L.	h	camphor; 1,8-cineole; a-pinene; endoborneol; camphene; verbenone	[28]
Rosmarinus officinalis L.	h	camphor, verbenone, and eucalyptol (1,8-cineole)	[102]
Rosmarinus officinalis L.	-	α-pinene; linalool; piperitone	[96]
Rosmarinus officinalis L.	m		[105]

 doi:10.20944/preprints202308.0422.v1

Rosmarinus officinalis L.	m, sub		[45]
Salvia officinalis L.	m		[90]
Salvia officinalis L.	h	thujone (trans); camphor; cineole,1,8	[109]
Salvia officinalis L.	h	cis-thujone; camphor; viridiflorol; 1,8-cineole; trans-thujone; camphene; manool	[29]
Salvia officinalis L.	h	camphor; thujone; isothujone	[102]
Satureja hortensis L.	h	carvacrol; gamma-terpinene; paracymene	[72]
Satureja hortensis L.	h	carvacrol; o-cymene; γ- terpinene; thymol	[112]
Satureja hortensis L.	m, sub		[45]
Satureja montana L.	h	carvacrol; p-cymene; borneol; thymoquinone; 1-octen-3-ol	[28]
Satureja montana L.	h	carvacrol; followed by its precursor p-cymene	[113]
Thymus leucotrichus Halácsy	h	thymol; p-cymene; g-terpinene; carvacrol	[28]
Thymus leucotrichus Halácsy	h	o-cymene; α-pinene; ç-terpinene; camphene	[73]
Thymus leucotrichus Halácsy	h	p-cymene; geraniol; thymol; carvacrol	[29]
Thymus leucotrichus Halácsy	p	thymol; p-cymene; linalool; caryophyllene oxide	[26]
Thymus leucotrichus Halácsy	h	thymol; p-cymene; γ-terpinene; caryophyllene oxide	[30]
Thymus leucotrichus Halácsy	h	thymol; p-cymene; γ-terpinene	[60]
Thymus leucotrichus Halácsy	se		[71]
Thymus leucotrichus Halácsy	m, sub	thymol; p-cymene; carvacrol; γ-terpinene	[45]
Thymus atticus Čelak.	h	carvacrol; o-cymene	[109]
Thymus atticus Čelak.	h	thymol; p-cymene; g-terpinene; carvacrol	[28]
Ziziphora clinopodioides Lam.	h	pulegone; piperitenone; isomenthone	[114]
Lauraceae			
Laurus nobilis L.	sfe		[115]
Laurus nobilis L.	se		[71]
Myrtaceae			
Myrtus communis L.	h	α-pinene;1,8-cineole	[79]
Oleaceae			
Olea europaea cv. Lechín de Sevilla	se		[71]
Olea europea cv. Arbequina	se		[71]
Olea europea cv. Cornicabra	se		[71]

 doi:10.20944/preprints202308.0422.v1

Olea europea cv. Empeltre	se		[71]
Olea europea cv. Erantoio	se		[71]
Olea europea cv. Picual	se		[71]
Papaveraceae			
Glaucium flavum Crantz	m		[78]
Poaceae			
Echinochloa crus-galli (L.) P. Beauv.	m	loliolide and tricin	[116]
Elytrigia repens (L.) Nevski	m, sub		[45]
Polygonaceae			
Polygonum aviculare L.	m, sub		[45]
Polygonum bistorta (L.) Samp.	m, sub		[45]
Pinaceae			
Cedrus atlantica (Endl.) Carrière	-	α-pinene; himachalane; β-himachalene	[96]
Picea abies (L.) H. Karst.	-	limonene; bornyl acetate; δ-cadinene; α-muurolol; δ-cadinol	[96]
Pinus pinea L.	se		[71]
Plantaginaceae			
Plantago albicans L.	m		[85]
Poaceae			
Echinochloa crus-galli (L.) P. Beauv.	m	loliolide and tricin	[116]
Punicaceae			
Punica granatum L.	se		[93]
Rosaceae			
Prunus dulcis (Mill.) D.A. Webb	-	fatty acids	[96]
Ranunculaceae			
Nigella sativa L.	m, sub		[45]
Rutaceae			
Ruta chalepensis L.	m		[104]
Ruta chalepensis L.			[117]
Ruta graveolens L.	se		[93]
Salicaceae			
Populus nigra	m	alkanes, sterols, aliphatic and triterpenoic alcohols, acidic compounds	[118]
Populus tremula L.	m		[119]
Solanaceae			
Hyoscyamus niger L.	m	vanillic acid	[120]

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Solanum villosum Mill.	m		[85]
Urticaceae			
Urtica dioica L.	m		[121]
Urtica dioica L.	m, sub		[45]
Urtica sp.	se		[71]
Verbenaceae			
Lantana camara L.	m		[117]
Zygophyllaceae			
Tribulus terrestris L.	m	flavonoids, saponins, tannins, steroids, cardiac glycosides, alkaloids, anthrequinones and terpenoids	[68]
Zygophyllum eichwaldii C.A.Mey.	m		[85]

*** Extraction methods**: m: maceration, se: Soxhlet extraction, h: hydrodistillation, sub: subcritical fluid extraction, p: purchased or provided, ultra: ultrasound assisted method, sfe: supercritical fluid extraction.

3. Extraction Methods and Determination of the Chemical Composition of Plant Extracts

The active compounds can be isolated from plant tissues with different extraction methods (Figure 1), using selective solvents. The extraction method is the first step to separate the active compounds from the raw material and a critical one to explore their bioactivity. The choice of the method is so crucial, that can affect the further separation as well as the chemical composition of the extracts [122].

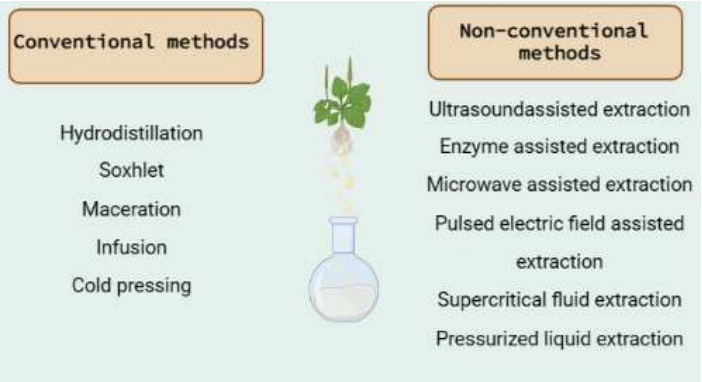

Figure 1. Conventional and non-conventional extraction methods [123,124].

Based on the literature data that has been compiled in Table 1, the most used methods are the following:

- Hydrodistillation: It is a traditional, simple method for extraction of active compounds and especially essential oils from plants. Even though it can be used in fresh plant materials, the material is preferred to be dried, in order to be preserved from enzymatic degradation [125]. As some volatile components may be lost at high extraction temperatures, this method cannot be used for thermolabile compounds [126]. In this method, water and oil are exclusively separated through condensation to retain all the essential properties of the plant [127]. So, it involves three main physicochemical processes: hydrodiffusion, hydrolysis, and heat decomposition [128]. Three types of hydrodistillation can be distinguished [123]: a) water distillation, b) water and steam distillation, and c) direct steam distillation. Umpiérrez et al. [129] report that the essential oils produced by different distillation methods did not differ in their chemical content in two Asteraceae plants. The hydrodistillation with the Clevenger type apparatus has been used in the

 doi:10.20944/preprints202308.0422.v1

majority of the extractions. In general, it is a widely utilized technique. It is a steam distillation technique, where the active compounds are extracted with the use of steam, generated outside the tank in a steam generator or in a boiler. It can determine the percentage of volatile oils present in the oil-bearing material [130]. The method is preferred because i) the released steam can easily be controlled and ii) no thermal decomposition of oil constituents occurs because the temperature does not exceed 100 °C. On the other hand, the required equipment has high cost [127].

- Soxhlet extraction or hot continuous extraction: It is a continuous extraction method with high extraction efficiency, that requires less time and solvent consumption than other methods (maceration or percolation) [131]. It is used for plant material that is partially soluble in the chosen solvent and for plant material with insoluble impurities [132]. There is also no need for filtration of the extract [125]. On the other hand, there is no possibility of shaking the device and the long extraction time may lead to the degradation of thermolabile compounds [133].
- Maceration: It is a solid-liquid extraction and one of the most widely used techniques in the medicinal and aromatic plant industry. It is a separation technique to remove a solute from a solid mixture with the help of a solvent [125]. It is an appropriate method for thermolabile plant material [132]. The success of the method depends on the solvent used, the plant part used as starting material and the extraction time. On the other hand, the large volume of the used solvents and the long extraction time are disadvantages of the method [127].

Figure 2. Solvents used for the extraction of different active compounds [123,124].

Especially the selection of solvent is crucial for the extraction. Solubility, selectivity, polarity, cost, and safety should be considered for the selection of the solvent [134]. Figure 2 shows different solvents used for the extraction of different active compounds from plant species. In general, methanol, ethanol, acetone, and water are preferred. Saaba et al. [135], analyzing the methanol, ethanol, acetone, and water extracts from different medicinal plants (such as *Juniperus phoenicea L.* and *Asphodelus microcarpus Salzm. & Viv.*), concluded that there were significant differences in the quantitative characterization of the different extracts depending on the solvent used. In this

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experiment, the acetone and methanol extracts seemed to be more promising. The solvents have different polarities, and this affects the content of the active compounds as well as their pesticidal activity. Water, methanol, and ethanol are used for the extraction of polar compounds (hydrophilic), whereas hexane and dichloromethane are used for the extraction of nonpolar compounds (lipophilic) [133,136].

Fractionation is also a widely used process that follows the extraction of raw material and aims to the isolation of specific compounds, belonging mainly in the same chemical category. It is a continuous process that ends after the isolation of the compound of interest and demands several solvents, which are added according to their polarity (from less to more polar) [125,134]. Fractionation was used for the isolation of alkaloids from *Sophora alopecuroides* L. extract [137], phenolic compounds from *Humulus lupulus* L. [95]-and isoflavones and flavones from *Retama raetam* [98].

Qualitative and quantitative analysis of phytochemicals presented in extracts/essential oils can be performed using chromatographic and identification techniques [132]. Mass spectrometry (MS) is a powerful analytical tool that is used to identify unknown compounds and has been applied to a very wide range of areas including biochemical sciences. Mass spectrometry provides abundant information for the structural elucidation of unknown compounds especially when tandem mass spectrometry (MS/MS) is applied [138]. Most of the scientific works reported herein have used Gas chromatography-mass spectroscopy (GC-MS) for the phytochemical analysis of the biopesticides [28, 75,79]. It is a combined analytical technique that plays an essential role in the phytochemical analysis of plant extracts containing biologically active compounds [139]. Advantages of the technique include i) the efficiency of gas chromatography separation, ii) the good qualitative information and high sensitivity provided by mass spectrometry (MS), and iii) the identification of the unknown compounds by comparison with library spectra [140].

It is worth mentioning that high performance liquid chromatography (HPLC) [90,94], liquid chromatography-mass spectroscopy (LC-MS) [87] as well as NMR [87,120] have also been employed for the identification of secondary metabolites. The chromatographic and identification techniques proved that the qualitative and quantitative variation of secondary metabolites in the same species depend on i) genetic factors, ii) environmental causes (light, temperature, soil water, soil fertility and salinity), iii) geographical origin iv) harvest stage, v) part of the plant vi) processing modalities, and vii) storage time [12,13,141].

4. Biological Activity of Plant Extracts and Essential Oils

Literature data indicate that plant extracts have a promising antimicrobial, insecticidal and herbicidal activity. Key findings of several recent studies focusing on the antimicrobial, insecticidal and activity of Mediterranean plant extracts and essential oils are presented in detail in Tables 2–4. Their activity was also examined to plant bacteria, viruses, nematodes as well as other pathogens (Table 5). Although numerous studies have evaluated the biological activities of the plant extracts and essential oils, in most cases the observed activities were not correlated with specific components. The biological activities were attributed to the synergistic effects of the different compounds [28]. Nevertheless, there were cases where the biological activity was correlated with specific compounds. Indicatively, γ-terpinene and myristicin were found to possess insecticidal activity and be effective on *C. quinquefasciatus* larvae [75].

It is also worth mentioning that in some cases the observed activity significantly varies for different and even the same targets between essential oils/extracts of the same plant. For example, Pavela et al. [75] investigated the essential oils of *Crithmum maritimum* L. of different geographical origins and observed a significant differentiation of their insecticidal activity, due to their phytochemical compositions. Furthermore, the activity of the essential oils of different parts of the plant was also found to vary. In a recent study, Zerkani et al. ǂ69] observed significant differences in antimicrobial activity from the essential oils derived from different parts of *Pistacia atlantica*.

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In addition, the same active compound has been reported to possess multiple biological activities. Theme oil having thymol as a major component was found to present antimicrobial properties by Ben Jabeur et al. [30]. Essential oils with thymol were also suggested as potential plant-based insecticidal agents [28]. Essential oils with carvacrol, piperitenone oxide as major compounds were also suggested [28,112] were also reported to possess insecticidal activity. Up to now, a variety of assays have been used to evaluate the biological activities, such as antimicrobial, insecticidal, herbicidal etc., of plant extracts and essential oils that are discussed in detail in the following sections.

Table 2. Recent studies on antimicrobial activity of Mediterranean plant extract/essential oils.

Fungus tested	Family	*Plant*	References
Alternaria alternata	Lamiaceae	*Lavandula canariensis* Mill.	[104]
	Rutaceae	*Ruta chalepensis* L.	
Alternaria solani	Lamiaceae	*Mentha ×piperita* L.	[105]
		Rosmarinus officinalis L.	
	Poaceae	*Echinochloa crus-galli* (L.) P. Beauv.	[116]
Alternaria spp.	Lamiaceae	*Thymus leucotrichus* Halácsy	[60]
		Hyssopus officinalis L.	
	Cupressaceae	*Juniperus communis* L.	
Botrytis cinerea	Cupressaceae	*Juniperus communis* L.	[59]
		Juniperus oxycedrus L.	
		Juniperus communis L. var. *saxatilis* Pall.	
	Lamiaceae	*Lavandula canariensis* Mill.	[104]
	Rutaceae	*Ruta chalepensis* L.	
	Lauraceae	*Laurus nobilis* L.	[115]
Cercospora kikuchii	Lamiaceae	*Lavandula dentata* L.	[103]
Cercospora sojina	Lamiaceae	*Lavandula dentata* L.	[103]
Colletotrichum spp.	Cupressaceae	*Juniperus communis* L.	[59]
		Juniperus oxycedrus L.	
		Juniperus communis L. var. *saxatilis* Pall.	
Cylindrocarpon pauciseptatum	Cupressaceae	*Juniperus communis* L.	[59]
		Juniperus oxycedrus L.	
		Juniperus communis L. *var. saxatilis* Pall.	
Fusarium culmorum	Salicaceae	*Populus tremula* L.	[119]
Fusarium oxysporum f. sp. *lycopersici*.	Lamiaceae	*Mentha ×piperita* L.	[105]
	Lamiaceae	*Rosmarinus officinalis* L.	

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Fusarium oxysporum	Lamiaceae	*Lavandula canariensis* Mill.	[104]
	Rutaceae	*Ruta chalepensis* L.	
	Lamiaceae	*Mentha ×piperita* L.	[105]
		Rosmarinus officinalis L.	
Fusarium spp.,	Cupressaceae	*Juniperus communis* L. var. *saxatilis* Pall.	[59]
		Juniperus oxycedrus L.	
		Juniperus communis L.	
Geotrichum citri-aurantii	Cistaceae	*Cistus albidus* L.	[54]
		Cistus creticus L.	
		Cistus crispus L.	
		Cistus ladanifer L.	
		Cistus laurifolius L.	
		Cistus monspeliensis L.	
		Cistus populifolius L.	
		Cistus salviifolius L.	
Mycosphaerella graminicola	Lamiaceae	*Thymus leucotrichus* Halácsy	[30]
Penicillium allii	Lamiaceae	*Origanum vulgare* L.	[110]
Phoma exigua	Lamiaceae	*Rosmarinus officinalis* L.	[45]
		Salvia officinalis L.	
		Satureja hortensis L.	
		Thymus leucotrichus Halácsy L.	
	Poaceae	*Elytrigia repens* (L.) Nevski	
	Polygonaceae	*Polygonum aviculare* L.	
		Persicaria bistorta (L.) Samp.	
	Ranunculaceae	*Nigella sativa* L.	
	Urticaceae	*Urtica dioica* L.	
Pythium ultimum	Lamiaceae	*Rosmarinus officinalis* L.	[105]
		Mentha ×piperita L.	
Rhizoctonia solani	Cupressaceae	*Juniperus communis* L.	[59]
		Juniperus oxycedrus L.	
		Juniperus communis L. var. *saxatilis* Pall.	
	Lamiaceae	*Mentha ×piperita* L.	[105]
		Rosmarinus officinalis L.	
Sclerotinia sclerotiorum	Apiaceae	*Cuminum cyminum* L.	[76]

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Septoria glycines	Lamiaceae	*Lavandula dentata* L.	[103]
Verticillium dahliae	Anacardiaceae	*Pistacia lentiscus* L.	[71]
	Apocynaceae	*Nerium oleander* L.	
	Araliaceae	*Hedera helix* L.	
	Asteraceae	*Dittrichia viscosa* (L.) Greuter	
	Brassicaceae	*Brassica rapa* L.	
		Diplotaxis erucoides (L.) DC.	
		Diplotaxis virgata (Cav.) DC.	
		Hirschfeldia incana (L.) Lagr.-Foss.	
	Cistaceae	*Cistus albidus* L.	
		Cistus ladanifer L.	
		Cistus laurifolius L.	
	Cupressaceae	*Juniperus communis* L.	
	Fagaceae	*Castanea sativa* Mill.	
	Junglandaceae	*Juglans regia* L.	
	Lamiaceae	*Marrubium vulgare* L.	
		Mentha x verticillata L.	
		Origanum vulgare L.	
		Rosmarinus officinalis L.	
		Salvia officinalis L.	
		Thymus leucotrichus Halácsy	
		Laurus nobilis L.	
	Oleaceae	*Olea europaea* cv. Lechín de Sevilla	
		Olea europea cv. Arbequina	
		Olea europea cv. Cornicabra	
		Olea europea cv. Empeltre	
		Olea europea cv. Frantoio	
		Olea europea cv. Picual	
	Papaveraceae	*Papaver rhoeas* L.	
	Pinaceae	*Pinus pinea* L.	
	Urticaceae	*Urtica* sp.	

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	Viburnaceae	*Sambucus nigra* L.	
Zymoseptoria tritici	Cannabaceae	*Humulus lupulus* L.	[95]
Alternaria alternata/ Alternaria solani/ Alternaria tenuissim/ Colletotrichum coccodes/ Fusarium oxysporum/ Fusarium sambucinum / Rhizoctonia solani/ Streptomyces scabiei	Apiaceae	*Carum carvi* L.	[45]
	Lamiaceae	*Thymus leucotrichus* Halácsy L.	
	Asteraceae	*Achillea millefolium* L.	
		Taraxacum officinale (L.) Weber ex F.H.Wigg	
	Cannabaceae	*Humulus lupulus* L.	
	Clusiaceae	*Hypericum perforatum* L.	
	Eqoisetaceae	*Equisetum arvense* L.	
	Lamiaceae	*Salvia officinalis* L.	
		Mentha ×piperita L.	
		Rosmarinus officinalis L.	
		Lavandula angustifolia Mill.	
		Satureja hortensis L.	
	Poaceae	*Elytrigia repens* (L.) Nevski	
	Polygonaceae	*Polygonum aviculare* L.	
		Persicaria bistorta (L.) Samp.	
	Ranunculaceae	*Nigella sativa* L.	
	Urticaceae	*Urtica dioica* L.	

4.1. Commonly Used Assays for Evaluating Antimicrobial Activity

Various methods are used to evaluate antimicrobial activity *in vitro*. Among them, the most common are agar dilution and disc diffusion methods. Agar dilution, otherwise referred to as poisoned food method, is the method of choice when estimating antifungal activity [142]. The method is based on preparing solid media and adding a desired concentration of the extract in them. A certain volume of the extract can be mixed before the autoclaved medium is poured on the petri dishes or spread on their surface once they have solidified [71,76,116]. Subsequently, a small agar plug (4-7 mm in diameter) from an active fungal culture is inverted, with the mycelial surface facing down, and inoculated at the center of the agar plate. The inhibition is estimated by measuring mycelial growth in optimum conditions and comparing it with a control sample [71]. One or multiple concentrations of the extract can be used during the assay. Different concentrations can be used to determine the potency of the antifungal effect, by measuring certain indices such as half maximal effective concentration (EC_{50} [76], the minimum inhibitory concentration (MIC) or the half inhibitory concentration (IC_{50}) of the extract [30,95]. Variations of the agar dilution method have been successfully employed to test the antifungal capacity of various extracts against plant pathogenic fungi such as *Verticillium dahliae* in olives [71], *Zymoseptoria tritici* in wheat [30,95], *Sclerotinia sclerotiorum* [76], *Fusarium oxysporum*, *Alternaria solani*, and *Pythium ultimum* in tomato [105,116], *Botrytis cinerea* [115], *Penicillium allii* [110], *Stemphylium vesicarium* [98], and *Geotrichum citri-aurantii* in decayed mandarin fruit [54]. Semerdjieva and colleagues used agar dilution to test the antifungal potential of essential oils against five fungal pathogens, including *Fusarium* sp. and *Rhizoctonia solani*

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strains isolated from stored potato, *Botrytis cinerea* from infected stored tomato, *Colletotrichum* sp. from anthracnose of bananas, and *Cylindrocarpon pauciseptatum* obtained from diseased grapevine [59]. Slight variations of the protocol involve inoculation of the agar containing the extract with a small volume from a liquid culture of the fungus [30,95] or with fungi-infected plant seeds [60], instead of an agar plug. Although the method is mostly used for fungal pathogens, Fu et al. [143] employed the agar dilution method to test the antibacterial potential of water extracts from aquatic weeds against 100 bacterial strains that were inoculated on the agar plates by streaking.

On the other hand, the disc diffusion method is mostly preferred when screening extracts for antibacterial activity *in vitro*. However, it can be used for testing antifungal activity as well [69]. This method is based on spreading an amount of bacterial or fungal suspension (or an agar plug from an active fungal culture) on solid media, placing small paper discs (5-6 mm in diameter) soaked with a microvolume of the extract (e.g., 3-5 µl), incubating the plates in ideal growth conditions, and measuring inhibition zones [142]. Disc diffusion was used to assess both antifungal and antibacterial activity of three subcritical carbon dioxide plant extracts from *Carum carvi*, *Thymus vulgaris*, and *Nigella sativa* [45]. The extracts were successful at inhibiting eight fungal pathogens, including *Fusarium*, *Alternaria*, *Colletotrichum*, *Rhizoctonia* and *Phoma* strains, as well as two bacterial phytopathogens belonging to the genera *Pectobacterium* and *Streptomyces* [45]. The study also employed another *in vitro* assay for testing antimicrobial activity, the agar-well diffusion method, which shares many similarities to the disc diffusion method. In its most common form, a volume (e.g., 50-250 µl) of the extract is applied in a central well (5-8 mm in diameter) on the agar plate, which was previously inoculated with the pathogen. Twenty-two water and water-glycol extracts were tested by this method for antimicrobial effect against the ten previously mentioned plant pathogens [45]. The disc diffusion method was used to assess the antifungal capacity of essential oils from *Lavandula dentata* against strains of *Cercospora kikuchii*, *Cercospora sojina*, and *Septoria glycines* [103], of pyroligneous acids identified in the bark of hybrid aspen trees against *Fusarium culmorum* [119], as well as of extracts from seven plant species collected from the island of Lampedusa, in Italy, against *Penicillium italicum*, *Aspergillus carbonarius*, and *Drechslera gigantea* [78]. It was also used to test the antibacterial effect of nano-suspensions of *Chrysanthemum coronarium* and *Azadirachta indica* against *Escherichia coli* and *Staphylococcus aureus* strains [89], (Hazafa et al., 2022), and of barnyard grass extracts against a tomato bacterial pathogen, *Pectobacterium carotovorum* [116]. Other applications of the method include screening against human pathogens. For instance, essential oils extracted from the aerial parts of *Origanum elongatum* were tested against nine pathogenic bacteria isolated from hospital patients [109], while essential oils from *Pistacia atlantica* were assayed against twelve human pathogens, nine bacterial and three fungal strains [69].

In vitro methods comprise the most common assays for antimicrobial screening since they are simple in terms of design and execution and provide useful and comprehensive results. On the other hand, *in vivo* and *in situ* assays are more challenging to set up and are thus less frequently used, but generally provide more reliable data. Such an *in situ* antimicrobial assay was carried out by Steglińska and colleagues on potatoes [45]. In brief, water, and subcritical carbon dioxide extracts (SCDE) from four plant species exhibited antifungal and antibacterial effects when they were applied on potatoes. The *in situ* assay included immersion of potatoes in the plant extracts, application of 20 µl of bacterial or fungal suspension in three cuts (5 mm in diameter and 5 mm deep), and measuring the infestation rate after 2 weeks of incubation [45]. A similar test was conducted by Karim and colleagues, who caused 2 mm deep and 3 mm wide wounds on mandarin fruit with sterile needles [54]. The cuts were inoculated with 30 ml of *Cistus* aqueous extracts and 20 ml from a *Geotrichum citri-aurantii* suspension. The incidence and severity of the fungal disease on the treated mandarin fruit was evaluated daily, for ten days [54]. Regarding antiviral activity, Hu et al. employed the half-leaf method to test the effect of nine compounds from the seeds of *Hyoscyamus niger* against a phytopathogenic virus, tobacco mosaic virus (TMV) [120]. The method is often used to test inactivation, protective and curative effects of extracts against the selected pathogen and is based on smearing half the surface of the leaf with the extract while leaving the other side with a control treatment. Depending on the type

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of effect that is being tested, the viral suspension is either mixed with the compounds and applied on the same side of the leaf or is inoculated on the whole surface of the leaf [144].

Table 3. Recent studies on insecticidal activity of Mediterranean plant extract/essential oils.

Insects tested	Family	Plant	Part	References
Acrobasis advenella	Lamiaceae	*Satureja hortensis* L.	aerial parts	[112]
Acromyrmex octospinosus	Apocynaceae	*Nerium oleander* L.	leaves	[83]
Aedes aegypti L.	Apiaceae	*Daucus carota* L.	umbels	[77]
Amblyseius swirskii	Lamiaceae	*Satureja hortensis* L.	aerial parts	[72]
Myzus persicae	Asteraceae	*Artemisia absinthium* L.	aerial parts	[36]
		Santolina chamaecyparissus L.	aerial parts	
		Tanacetum vulgare L.	aerial parts	
	Compositeae	*Achillea millefolium* L.	aerial parts	
	Fabaceae	*Sophora alopecuroides* L.	aerial parts	[99]
	Lamiaceae	*Origanum syriacum* L. subsp. *syriacum*	leaves	[25]
	Lamiaceae	*Satureja montana* L.	leaves and flowers	[113]
experimental model of aphids' nervous system	Lamiaceae	*Lavandula angustifolia* Mill.	aerial parts	[102]
		Satureja montana L.	aerial parts	
		Salvia officinalis L.	aerial parts	
Aphis craccivora	Resedaceae	*Ochradenus baccatus* Delile	leaves	[90]
	Asteraceae	*Pulicaria crispa* (Forssk.) Oliv. (Forssk.) Oliv.	leaves	
	Lamiaceae	*Salvia officinalis* L.	leaves	
Apis mellifera	Asteraceae	*Artemisia absinthium* L.	aerial parts	[129]
Aphis citricola	Fabaceae	*Sophora alopecuroides* L.	aerial parts	[99]
Macrosiphum rosirvorum	Fabaceae	*Sophora alopecuroides* L.	aerial parts	[99]

 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
Sitobion avenae	Cupressaceae	*Juniperus communis* L.		[59]
Brevicoryne brassicae		*Juniperus oxycedrus* L.		
Brassicogethes aeneus		*Juniperus communis* var. *satilis* Pall.		
Callosobruchus maculatus	Anacardiaceae	*Pistacia atlantica* Desf.	fruits, leaves and gum	[70]
		Pistacia khinjuk Stocks	fruits and leaves	
Ceratitis capitata	Labiatae	*Origanum elongatum* (Bonnet) Emb. & Maire	aerial parts	[109]
	Anacardiaceae	*Pistacia atlantica* Desf.		[69]
	Lamiaceae	*Mentha suaveolens* Ehrh.		[145]
		Salvia officinalis L.		
		Thymus atticus Čelak.		
Chaitophorus populialbae	Dennstaedtiaceae	*Pteridium aquilinum* (L.) Kuhn (L.) Kuhn	leaves	[97]
Chrysoperla carnea	Lamiaceae	*Salvia officinalis* L.	leaves	[90]
	Resedaceae	*Ochradenus baccatus* Delile	leaves	
	Asteraceae	*Pulicaria crispa* (Forssk.) Oliv.	leaves	
Culex pipiens L.	Apiaceae	*Daucus carota* L.		[77]
Culex quinquefasciatus	Apiaceae	*Smyrnium olusatrum* L.	umbels	[81]
		Helosciadium nodiflorum (L.) W.D.J. Koch	aerial parts	
	Chenopodiaceae	*Chenopodium murale* (L.) S. Fuentes & al.	whole plant	[68]
	Amaranthaceae	*Achyranthes aspera* L.	whole plant	

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 August 2023 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
	Zygophyllaceae	*Tribulus terrestris* L.	whole plant	
	Convolvulaceae	*Convolvulus arvensis* L.	whole plant	
	Apiaceae	*Crithmum maritimum* L.	aerial parts, leaves, flowers and seeds	[75]
	Lamiaceae	*Ziziphora clinopodioides* Lam.	aerial parts	[114]
Culex restuans Theobald	Apiaceae	*Daucus carota* L.	umbels	[77]
Cydia pomonella L.	Cannabaceae	*Humulus lupulus* L.		[67]
Dendrolimus pini L.	Brassicaceae	*Sinapis alba* L.		[67]
Diaphorina citri	Asteraceae	*Artemisia absinthium* L.	leaves and flowers	[146]
Epicauta atomaria	Lamiaceae	*Lavandula dentata* L.	leaves and green stems	[103]
Harmonia axyridis	Lamiaceae	*Origanum syriacum* L. subsp. *syriacum*	leaves	[25]
Leptinotarsa decemlineata	Lamiaceae	*Phlomis tuberosa* L.	stems, leaves, flowers	[44]
	Apiaceae	*Bifora radians* M. Bieb.	leaves and stems	
	Apiaceae	*Heracleum platytaenium* Boiss.	leaves and stems	
	Acanthaceae	*Acanthus dioscoridis* L.	stems, leaves, flowers	
	Cannabaceae	*Humulus lupulus* L.	cone	
	Asteraceae	*Achillea millefolium* L.	stems, leaves, flowers	
	Lamiaceae	*Satureja montana* L.	leaves and flowers	[113]
	Asteraceae	*Santolina chamaecyparissus* L.	aerial parts	[91]
	Lamiaceae	*Hyssopus officinalis* L.	aerial parts	
	Lamiaceae	*Lavandula* × *intermedia* Emeric ex Loisel.	aerial parts	
Macrosiphum euphorbiae	Apiaceae	*Foeniculum vulgare* Mill. Mill.		[80]

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 August 2023 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
	Apiaceae	*Pimpinella anisum* L.		
Musca domestica	Lamiaceae	*Origanum syriacum* L. subsp. *syriacum*	leaves	[25]
Phthorimaea operculella	Plantaginaceae	*Plantago albicans* L.		[85]
	Solanaceae	*Solanum villosum* Mill.		
	Zygophyllaceae	*Zygophyllum eichwaldii* C.A.Mey.		
Rhopalosiphum maidis	Apiaceae	*Foeniculum vulgare* Mill.		[79]
	Myrtaceae	*Myrtus communis* L.		
Rhopalosiphum padi	Cupressaceae	*Juniperus communis* L.		[59]
	Cupressaceae	*Juniperus oxycedrus* L.		
	Cupressaceae	*Juniperus pygmaea*		
	Lamiaceae	*Hyssopus officinalis* L.	aerial parts	[91]
	Lamiaceae	*Lavandula × intermedia* Emeric ex Loisel.	aerial parts	
	Asteraceae	*Santolina chamaecyparissus* L.		
Rhyzopertha dominica	Asteraceae	*Glebionis coronaria* (L.) Spach		[89]
Sitophilus oryzae	Lamiaceae	*Mentha longifolia* (L.) Huds.		[147]
Sitophilus zeamais	Lamiaceae	*Lavandula dentata* L.	leaves and green stems	[103]
Spodoptera exigua	Brassicaceae	*Sinapis alba* L.		[67]
Spodoptera frugiperda	Fabaceae	*Ulex europaeus* L.	leaves and flowers	[93]
	Punicaceae	*Punica granatum* L.	fruit peel	
	Rutaceae	*Ruta graveolens* L.	leaves	
	Boraginaceae	*Glandora prostrata* (Loisel.) D.C.Thomas	leaves and flowers	

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 August 2023 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
	Labiatae	*Origanum majorana* L.	leaves and stems	[107]
	Lamiaceae	*Nepeta cataria* L.	leaves and stems	
		Origanum vulgare L.	leaves and stems	
	Lythraceae	*Punica granatum* L.	fruit peel	
Spodoptera littoralis	Labiatae	*Origanum virens* Hoffmanns. & Link	aerial parts	[28]
	Lamiaceae	*Lavandula angustifolia* Mill.	aerial parts	
	Lamiaceae	*Satureja montana* L.	aerial parts	
	Lamiaceae	*Thymus leucotrichus* Halácsy	aerial parts	
	Lamiaceae	*Thymus atticus* Čelak.	aerial parts	
	Lamiaceae	*Mentha ×piperita* L.	aerial parts	
	Lamiaceae	*Satureja montana* L.	aerial parts	
	Lamiaceae	*Mentha spicata* L.	aerial parts	
	Lamiaceae	*Mentha suaveolens* Ehrh.	aerial parts	
	Asteraceae	*Artemisia inculta* Delile	aerial parts	
	Lamiaceae	*Origanum syriacum* L. subsp. *syriacum*	aerial parts	[25]
	Lamiaceae	*Satureja montana* L.	aerial parts	[91]
	Lamiaceae	*Hyssopus officinalis* L.	aerial parts	
	Lamiaceae	*Lavandula × intermedia* Emeric ex Loisel.	aerial parts	
	Asteraceae	*Santolina chamaecyparissus* L.	aerial parts	
Tetranychus cinnabarinus	Asteraceae	*Artemisia capillaris* Thunb.		[148]
Tetranychus turkestani	Lamiaceae	*Mentha longifolia* (L.) Huds. L.		[149]
	Lamiaceae	*Rosmarinus officinalis* L.		
Tetranychus urticae	Lamiaceae	*Satureja hortensis* L.	aerial parts	[72]

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 August 2023 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
	Apiaceae	*Anethum graveolens* L.	aerial parts	
	Boraginaceae	*Onosma visianii* Clementi	roots	[94]
	Caryophyllaceae	*Saponaria officinalis* L.		[75]
Thrips tabaci	Lamiaceae	*Satureja montana* L.	leaves and stems	[111]
Trialeurodes vaporariorum	Asteraceae	*Artemisia absinthium* L.	aerial parts	[129]
Tribolium castaneum	Cupressaceae	*Juniperus phoenicea* L.	leaves	[58]
		Cupressus sempervirens L.	leaves	
	Asphodelaceae	*Asphodelus microcarpus* Salzm. & Viv.	leaves	
	Lamiaceae	*Mentha rotundifolia* (L.) Huds	leaves	
	Lamiaceae	*Lavandula dentata* L.	leaves and green stems	[103]
	Asteraceae	*Glebionis coronaria* (L.) Spach	leaves and flowers	[89]
	Lamiaceae	*Mentha spicata* L.	plant samples	[106]
Tribolium confusum	Lamiaceae	*Lavandula angustifolia* Mill.		[96]
	Lamiaceae	*Mentha ×piperita* L.		
	Lamiaceae	*Satureja montana* L.		
	Pinaceae	*Picea abies* (L.) H. Karst.		
	Rosaceae	*Prunus dulcis* (Mill.) D.A. Webb		
Trichoplusia ni	Lamiaceae	*Thymus leucotrichus* Halácsy		[26]
Trogoderma granarium	Rutaceae	*Ruta chalepensis* L.	aerial parts	[117]
	Verbenaceae	*Lantana camara* L.	aerial parts	
	Apocynaceae	*Calotropis procera* (Aiton) W.T.Aiton	leaves	[82]
Tuta absoluta	Asteraceae	*Tanacetum vulgare* L.	flowers, leaves, buds	[92]

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 August 2023 doi:10.20944/preprints202308.0422.v1

Insects tested	Family	Plant	Part	References
	Lamiaceae	*Mentha suaveolens* Ehrh.		[145]
	Lamiaceae	*Salvia officinalis* L.		[109]
	Lamiaceae	*Thymus atticus* Čelak.		
	Anacardiaceae	*Pistacia atlantica* Desf.	leaves, fruits, and barks	[69]
	Asteraceae	*Tanacetum vulgare* L.	flowers, leaves, buds	[92]

4.2. Bioassays for Determining Pesticidal or Repellent Activity

Plant extracts can be submitted to a variety of assays to evaluate their insecticidal, acaricidal, nematocidal, or repellent potential, as well as their effect on oviposition. Standardized techniques include topical application, residual or surface contact, immersion in the extract or in a solution containing the extract, feeding bioassays, and fumigation [80,150]. Usually, the selected assay takes into consideration the unique biology of each pest, or its developmental stage, since the egg and larval stages have different morphological and biological characteristics than the adults.

Among the previously mentioned techniques, topical application can be used for bioassays in most developmental stages. The technique is based on applying microvolumes of the extract directly on the body of the insect with a micropipette or a microsyringe [150]. It was used successfully for larvae of the lepidopteran *Spodoptera littoralis*. Different concentrations of *Origanum syriacum* subsp. *syriacum* extract were mixed with 1 µl of acetone and each solution was applied on the dorsal region of 80 larvae per dose [25]. Insecticidal bioassays using topical application of extracts with a microsyringe was similarly performed on the dorsal region of *Spodoptera frugiperda* larvae [107]. Topical application tests can be performed also on adult individuals. In this case however, since adults of certain insects display high motility or flying ability, as a first step before the topical application of the extract, the insects are anaesthetised with CO_2 or on ice [25,88,151]. For instance, female *Musca domestica* flies were first anaesthetised and then treated with different doses of *Origanum syriacum* subsp. *syriacum* extracts by applying a microvolume of the extract on the pronotum of the flies and measuring the effect after 24 hours [25]. Topical application methods have been used to assay multiple insect species, such as *Pectinophora gossypiella*, *Thaumatotibia leucotreta*, *Helicoverpa armigera*, *Myzus persicae*, *Aphis craccivora*, *Aphis citricola*, *Aedes aegypti*, *Diaphorina citri*, *Tribolium castaneum*, *Trichoplusia ni*, *Brassicogethes aeneus* etc. [26,58,73,88,99,146,151]. In the case of *Trichoplusia ni* larvae, an injection assay was also performed, with one microliter of test solution injected into the ventral hemocoel [26].

On the other hand, during residual contact techniques, individuals or groups of target organisms are exposed to residues of the bioactive compounds. The compounds are usually added uniformly on natural (e.g., leaves, fruit, inflorescences) or artificial (e.g., filter discs) surfaces, and the specimens are placed on them [150]. Such a residual contact assay was applied by Alkan and Gökçe [44] on egg masses of the Colorado potato beetle *Leptinotarsa decemlineata*. The eggs that were oviposited on potato leaflets were sprayed with 20 µl of six plant extracts to examine their ovicidal effect. The leaflets were then placed in petri dishes and egg mortality was recorded for 7 days [44]. Residual spraying was also used to apply plant essential oils on adult aphids (*Myzus persicae*) [36]. Other surface contact techniques, that did not employ spraying, were used to determine the acaricidal efficacy of different concentrations of an extract from *Onosma visianii* roots [94]. The mite that was subjected to the treatment belonged to the species *Tetranychus urticae*. A pipette was used to apply 20 µl of the various dilutions on one side of bean leaf discs (sized 2 cm^{-2}), which were then placed on

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agar containing plates. Various developmental stages of the mites were assayed. Adult females, nymphs or eggs were transferred on the discs and incubated at fixed temperature and light conditions for 24 hours or up to five days after the treatment. Thus, this assay, with minor modifications for each case, was used to assess adult mortality, the number of oviposited eggs for live females, and the hatchability of eggs [94]. A similar study was carried out for *Saponaria officinalis*-synthesized silver nanocrystals against *Tetranychus urticae* [75]. Surface toxicity was also used to assess the larvicidal activity of *Tagetes minuta* essential oils to *Lucilia cuprina* flies. The applied protocol was based on transferring 3rd instar larvae of the fly in glass vials with filter papers impregnated with different dilutions of the essential oils [152]. Various residual or surface contact bioassays, with certain modifications in their protocols, were used to test the bioactivity of a variety of plant extracts and essential oils against eggs, larvae and adult specimens of insects and mites [70,72,74,82,87,89,90,92,97,101,112,129,146,147,149,153,154,155,156,157,158]. For instance, Erdogan and Mustafa, dipped tomato leaf discs in the test solutions instead of pipetting a volume on their surface, and then placed *Tuta absoluta* larvae on them [92]. Surface contact bioassays can be performed not only on a laboratory scale but also on a larger scale. For instance, extracts from leaves of *Agave americana* were used against the hemipteran *Brevicoryne brassicae* in field experiments performed at a cabbage farm. The application of the extracts was done by spraying parts of the leaves and the centre of the adult plant [155].

Repellency, rather than acute toxicity or pest mortality, may also be assessed with modified surface contact methods. Typical repellency assays use filter papers which are treated with the extract in one half and the respective solvent in the other half and are subsequently placed in petri dishes with the test samples [106]. Such repellency bioassays were carried out for larvae of the khapra beetle, *Trogoderma granarium* [82], and adults of *Tribolium castaneum* [106]. Ilyas and colleagues, on the other hand, treated guava fruits by immersing them into plant extract solutions. The treated fruits were subsequently provided to adult *Bactrocera zonata* flies that were kept in cages, and the number of individuals that would settle on the fruits would be recorded for 5 hours per day, for two days [153]. Mangang and colleagues, also used a more sophisticated system, termed insect management unit, to study the repellent properties of packaging material [106]. Pourya et al. also used an arena to perform repellency bioassays on adult *Callosobruchus maculatus* beetles [70]. The arena consisted of three plastic chambers that were connected by small tubings. The beetles were placed in the central chamber, the control cowpeas treated only with solvents in the first test chamber, and the cowpeas that were treated with different concentrations of *Pistacia* essential oils in the second test chamber [70].

Immersion techniques are especially suitable for developmental stages that take place within an aquatic environment, such as eggs or larvae of certain species. Therefore, immersion assays were performed on larvae of *Culex quinquefasciatus* mosquitoes [68,75,81,114]. The larvae were placed in 250 ml of solution containing 249 ml of distilled water and 1 ml of the essential oils or their mixture (six different dosages were tested for each compound) and their mortality was recorded after 24 h of exposure to the treatment [81]. Similar approaches were used in other studies featuring larvae of other mosquito species, such as *Culex pipiens*, *Culex restuans*, *Aedes aegypti*, *Aedes albopictus,* and *Anopheles gambiae* [77,82,88,159,160]. Musso and colleagues, used immersion techniques to study the larvae of the nematode *Panagrolaimus rigidus* [108]. Briefly, they placed in each well of a 96-well microplate, 100 μl of suspension containing approximately 100 larvae. Then, they added 100 μl of essential oil solutions isolated from *Nepeta* plant species and incubated the microplates at 20°C. Nematocidal activity was estimated by counting mobile and immobile roundworms using an optical microscope [108]. Immersion bioassays can be also performed to test the activity of extracts on insect eggs [161]. In that case, eggs of the lepidopteran *Conopomorpha sinensis* were submerged in two different concentrations of various plant extracts for 10 seconds, and their hatching rate was measured for two days [161]. The use of solid formulations against the potato tuber moth *Phthorimaea operculella* can be considered a modified case of immersion methods [85]. The process was based on crude extracts that were mixed with talcum powder (magnesium silicate) as inert carrier substrate.

 doi:10.20944/preprints202308.0422.v1

Moths were completely covered with the powdered extract which was firmly attached to their cuticle. Mortality and other biological parameters of the moths were recorded after the application of the powder [85]. Immersion based assays were carried out to study the nematocidal activity against other species of nematodes, such as *Meloidogyne incognita* [121], and *Meloidogyne javanica* [91,113], as well as the acaricidal activity against *Tetranychus cinnabarinus* mites using the slip-dip method [148].

Feeding bioassays were performed against adult aphids of the species *Myzus persicae*. In this case, different concentrations of *Origanum syriacum* subsp. s*yriacum* extracts were applied on cabbage, and 4 groups of 50 individuals were left to feed on it. Mortality was recorded 48 hours after the application of the treatment [25]. Similar feeding assays were conducted for the leaf-cutting ants *Acromyrmex octospinosus*, using extracts from *Mammea americana* seeds, *Nerium oleander* and *Nicotiana tabacum* leaves [83]. The insecticidal activity of *Brassica alba* mustard oil against the lepidopteran species, *Cydia pomonella*, *Dendrolimus pini*, and *Spodoptera exigua* [67], as well as of *Eucalyptus* essential oils on *Sitophilus oryzae* and *Sitophilus granarius* [162], were also assessed by feeding bioassays. Feeding inhibition caused by *Satureja montana* essential oils was measured for *Spodoptera littoralis* larvae, *Myzus persicae* and *Leptinotarsa decemlineata* adults. The antifeedant activity was calculated by measuring the consumption of treated leaf discs and comparing it with the controls [113]. Different concentrations of extracts can be mixed and tested not only with a natural host, but also with artificial larval diets. Such was the case of *Spodoptera frugiperda* (fall armyworm) larvae that were submitted to various concentrations of extracts from the aerial parts of *Senna crotalarioides* plants [163]. Similar feeding inhibition assays were conducted with other extracts isolated from various plant species, such as *Hyssopus officinalis*, *Lavandula intermedia*, and *Santolina chamaecyparissus* [91], fourteen plant species belonging to the families Asteraceae and Lamiaceae [28], or with trans-anethole compounds from various Apiaceae species [156].

Fumigant bioassays can be conducted for volatile organic compounds. For instance, volatile essential oils isolated from bitter fennel (*Foeniculum vulgare*) and green anise (*Pimpinella anisum*) were tested for their insecticidal activity against *Macrosiphum euphorbiae* aphids that infest tomatoes [80]. The tested essential oils were applied on filter papers, and the experiment was conducted on a small scale (only on tomato leaflets), and on a large scale, both with whole plants and at greenhouse level [80]. A different setup was used to test the insecticidal activity of lemongrass and rosemary essential oils against onion thrips *Thrips tabaci*. Small *Allium schoenoprasum* seedlings with approximately 20 leaves were inserted separately into 50 ml test tubes. One milliliter glass tubes containing the essential oils were placed in each test tube along with ten adult thrips, for three days, and mortality rate was calculated [111]. Other cases of fumigant bioassays with plant extracts and volatile essential oils have also been documented [70,74,149,162].

It is crucial for novel biopesticides to show high specificity and activity only against their intended target-pests. For that reason, similar bioassays can be executed to assess the safety of the compounds against non-target organisms, such as the ladybug *Harmonia axyridis*, *Eisenia fetida* earthworms, the green lacewing *Chrysoperla carnea*, honeybees, or *Trichogramma pretiosum* hymenoptera [25,90,107,129]. Non-target organisms may also include predatory mites, such as the species *Amblyseius swirskii*, which is widely used as a natural enemy for biological control of small pest species including mites, thrips, and whiteflies [72]. Similarly, Pino-Otín and colleagues assessed the ecotoxicological impact of a biopesticide from *Artemisia absinthium* on the soil microbial communities, the earthworm *Eisenia fetida* and the plant *Allium cepa*. The changes in microbial communities were assessed with metagenomic amplicon sequencing of the 16S rRNA, and the toxicity tests on the onion plant were conducted on young bulbs. For the nematocidal assay, they estimated mortality, by placing 10 adult earthworms on 500 gr of soil in 1 L plastic containers treated with different concentrations of the aqueous extract [164].

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Table 4. Recent studies on herbicidal activity of Mediterranean plant extract/essential oils.

Weeds tested	Family	Plant	Part	References
Abutilon theophrasti	Compositeae	*Solidago virgaurea* L.	leaves and flowers	[29]
	Lamiaceae	*Melissa officinalis* L.	leaves	
		Salvia officinalis L.	leaves and flowers	
		Thymus leucotrichus Halácsy	areal part	
Amaranthus powellii	Brassicaceae	*Sinapis alba* L.	seeds	[66]
Amaranthus retroflexus L.	Asteraceae	*Cynara cardunculus* L.	leaves	[86]
Amaranthus spinosus	Poaceae	*Echinochloa crus-galli* (L.) P. Beauv.	leaves	[116]
Anagallis arvensis L.	Asteraceae	*Cynara cardunculus* L.	leaves	[86]
Brassica rapa	Salicaceae	*Populus tremula* L.	bark mass, including both inner and outer layers	[119]
Capsicum annuum	Lamiaceae	*Calamintha menthifolia* Host		[101]
Cyperus iria L.	Poaceae	*Echinochloa crus-galli* (L.) P. Beauv.	leaves	[116]
Echinochloa crus-galli (L.) P. Beauv.	Apiaceae	*Carum carvi* L.	seeds	[46]
	Apiaceae	*Mentha×piperita* L.		
Lolium perenne	Asteraceae	*Santolina chamaecyparissus* L.	aerial parts	[91]
	Lamiaceae	*Hyssopus officinalis* L.	aerial parts	
		Lavandula × intermedia Emeric ex Loisel.	aerial parts	
Melilotus officinalis L.	Cupressaceae	*Juniperus excelsa* M. Bieb.	leaves	[61]
	Cupressaceae	*Juniperus sabina*	leaves	[61]
Myosotis arvensis	Cupressaceae	*Juniperus excelsa* M. Bieb.	leaves	[61]
		Juniperus sabina	leaves	
Orobanche cumana	Fabaceae	*Retama raetam* (Forssk.) Webb	aerial parts	[98]
Portulaca oleracea L.	Asteraceae	*Cynara cardunculus* L.	leaves	[86]
Setaria viridis	Brassicaceae	*Sinapis alba* L.	seeds	[66]
Sitobion avenae		*Juniperus communis* L.		[61]

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	Cupressaceae	*Juniperus oxycedrus* L.		
		Juniperus communis var. *saxatilis* Pall.		
Solanum nigrum	Lamiaceae	*Clinopodium menthifolium* (Host)		[101]
Stellaria media (L.) Vill.	Asteraceae	*Cynara cardunculus* L.	leaves	[86]
Trigonella besseriana Ser.	Cupressaceae	*Juniperus excelsa*	leaves	[61]
		Juniperus sabina L.	leaves	
Plants phytotoxicity tested	**Family**	**Plant**	**Part**	**References**
Solanum lycopersicum	Lamiaceae	*Prasium majus* L.		[78]
	Papaveraceae	*Glaucium flavum* Crantz		
	Apiaceae	*Daucus lopadusanus* Tineo		
	Asclepiadaceae	*Periploca angustifolia* Labill.		
	Asteraceae	*Echinops spinosissimus* Turra		
	Chenopodiaceae	*Atriplex halimus* L.		
	Clusiaceae	*Hypericum aegypticum* L.		
	Asteraceae	*Artemisia absinthium* L.	aerial parts	[129]
Arabidopsis thaliana	Juncaceae	*Juncus compressus* Jacq.		[100]

4.3. Bioassays for Determining Herbicidal Activity

Based on the average pesticide consumption of the EU-27 Member States during the period 2010–2019, herbicides represent more than 30% of all pesticides used in the EU [165], while worldwide herbicides account for 50 % of all pesticides used of which >75% are used in developed countries [13]. The reduction of herbicide use premises the adoption of suitable, alternative weed management strategies. However, farmers tend to focus on the short-term economic benefits, while the agroecological benefits of herbicide reduction are long-term oriented. In contrast to the use of synthetic herbicides, bioherbicides are an ecologically sustainable alternative that is a priority in the EU. These eco-friendly herbicides can be subdivided into microbial bioherbicides and bio-derived (biochemical) bioherbicides. Microbial bioherbicides are made of bacteria, fungi or viruses, being in their active form (liquid formulation) or in dormant form (dry formulation). Bio-derived bioherbicides have as active ingredients natural molecules extracted in most cases from plants. However, botanical products can be "heterogeneous" due to the presence of mixtures of bioactive components either from the same or from purposefully mixed botanical sources. Physical analytical methods, such as chromatography, are useless for this purpose as they are usually insensitive to the chemical complexities found in crude botanical extracts. Most often a desired biological response is due to not one but a mixture of bioactive plant components, and the relative proportions of single bioactive compounds can vary from batch to batch while the bioactivity remains within tolerable limits. Thus, physical, or chemical analysis of a single component in such mixtures is not completely satisfactory [166]. Thus, the isolation of plant allelopathic substances and the evaluation of their phytotoxic effects can lead to the discovery of new natural herbicides. For the above reasons, a decisive factor in the "discovery of bioherbicides" is the evaluation of the herbicidal activity of plant extracts by bioassays.

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The herbicidal activity of plant extracts evaluation can be estimated either in laboratory scale using *in vitro* assays, or in the field by pre- and postemergence assays. The *in vitro* assay evaluates the seed germination in petri plates. The inhibitory effects of the extract on weed seeds are determined by counting the germinated seeds (percent of germination), the root length of germinated seeds, the sprout length etc. Firstly, it is crucial that the seed surface is sterilized to avoid possible inhibition of germination caused by fungal or bacterial toxins. The seeds are placed in a filter paper soaked by the extract [78] or covered by a soaked filter paper [61]. One or multiple concentrations of the extract can be used during the assay [29]. The dishes are sealed with parafilm to avoid evaporation of the extract and incubated in certain conditions of temperature and photoperiod. Variations of the method have been successfully employed to test extracts from various Mediterranean species against weeds such as *Melilotus officinalis* L., *Myosotis arvensis* (L.) Hill and Trigonella *besseriana* Ser. [61] or *Amaranthus retroflexus* L. and *Portulaca oleracea* L., *Stellaria media* (L.) Vill. and *Anagallis arvensis* [86]. The method can also be applied in germinating seedlings [119]. On the other hand, evaluation of the herbicidal activity can also be estimated in the field in pre- and postemergence assays. Morra et al. [66] evaluated the activity of *Sinapis alba* extract to the seeds of *Amaranthus powellii* and *Setaria viridis*. In preemergence assays the solution of the extract is applied to the surface of the pot, while in postemergence assays the extract either is sprayed or watered [116]. In preemergence assays the emerged live seedlings, the plant height and the dry weight are recorded, while in postemergence assays live plants per pot, plant height and dry weight are determined [66].

Table 5. Recent studies on bacterial, antiviral and nematicidal activity of Mediterranean plant extract/essential oils.

Control	Target tested	Family	Plant	Part	References
Bacteria	*Clavibacter michiganensis*	Asteraceae	*Achillea ptarmica* L.	aerial parts	[84]
			Achillea millefolium L.	aerial parts	
			Arctium lappa L.	aerial parts	
			Bidens tripartite L.	aerial parts	
			Carduus acanthoides L.	aerial parts	
			Carduus nutans subsp. *leiophyllus* (Petrović) Stoj. & Stef.	aerial parts	
			Centaurea cyanus L.	aerial parts	
			Centaurea jacea L.	aerial parts	
			Centaurea scabiosa L.	aerial parts	
			Cirsium arvense (L.) Scop.	aerial parts	
			Echinops ritro L.	aerial parts	
			Gnaphalium uliginosum L.	aerial parts	
			Pentanema britannica (L.) D.Gut.Larr., Santos-Vicente, Anderb., E.Rico & M.M.Mart.Ort.	aerial parts	
			Sonchus arvensis L.	aerial parts	
			Tripleurospermum inodorum (L.) Sch. Bip.	aerial parts	

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Control	Target tested	Family	Plant	Part	References
		Compositae	*Leontodon hispidus* L.	aerial parts	
			Silybum marianum (L.) Gaertn.	aerial parts	
	Pectobacterium carotovorum	Apiaceae	*Carum carvi* L.	seeds	[45]
		Asteraceae	*Achillea millefolium* L.	stems, leaves, flowers	
		Asteraceae	*Taraxacum officinale* F.H. Wigg. subsp. *officinale*	leaves, stems	
		Cannabaceae	*Humulus lupulus* L.	inflorescences	
		Clusiaceae	*Hypericum perforatum* L.	root	
		Eqoisetaceae	*Equisetum arvense* L.	leaves, stems	
		Lamiaceae	*Lavandula angustifolia* Mill.	flower buds	
			Mentha ×piperita L.	leaves, stems	
			Rosmarinus officinalis L.	leaves, stems	
			Salvia officinalis L.	stems	
			Satureja hortensis L.	leaves, stems	
			Thymus leucotrichus Halácsy	seeds	
		Poaceae	*Echinochloa crus-galli* (L.) P. Beauv.	leaves	[116]
		Poaceae	*Elymus repens* (L.)	leaves, stems	[45]
		Polygonaceae	*Polygonum aviculare* L.	leaves, stems	
		Polygonaceae	*Polygonum bistorta* L. Samp.	leaves, stems	
		Ranunculaceae	*Nigella sativa* L.	seeds	
		Urticaceae	*Urtica dioica* L.	stems	
Virus	Tobacco Mosaic Virus	Solanaceae	*Hyoscyamus niger* L.	seeds	[120]
Clitellata	*Eisenia fetida*	Asteraceae	*Artemisia absinthium* L. (var. Candial)		[164]
		Lamiaceae	*Origanum syriacum* L. subsp. *syriacum*	leaves	[25]

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Control	Target tested	Family	Plant	Part	References
	Panagrolaimus rigidus	Lamiaceae	*Nepeta curviflora* Webb & Berthel.	flowering tops, seeds, and leaves	[108]
		Lamiaceae	*Nepeta nuda* L. ssp. *pubescens*	flowering tops, seeds, and leaves	
Nematodes	*Meloidogyne incognita*	Urticaceae	*Urtica dioica* L.	whole plant	[121]
	Meloidogyne javanica	Lamiaceae	*Satureja montana* L.	leaves and flowers	[113]

5. Toxicity and Safety Concerns

In general, biopesticides have nontoxic ways of action and are more selective in their targets than synthetic chemical pesticides [13]. However, some compounds in high doses may provoke toxic effects in nontarget organisms. Several suggestions, guidelines, regulations, and directives about biopesticides and their regulation and registration process have been published by agencies worldwide. For example, Regulation (EC) No 1107/2009 requires analysis of impurities from the plant protection products by toxicological and environmental testing [167]. Moreover, FAO (2017) with its guidelines for the registration of microbial, botanical and semiochemical pest control agents for plant protection and public health uses, requires (if previous assessments are not available or sufficient) acute and/or longer-term studies [3]. US EPA (2012) indicates that the limit dose for most pesticides is 25 μg of active ingredient per *Apis mellifera* L. honeybee [168].

In Table 6 indicative recent studies that have conducted toxicity assessments of plant extracts/essential oils are compiled. Recently, Di Lecce et al. [78] studied the potential toxic effects of extracts from seven plant species. The authors observed toxicity of some extracts towards hepatocarcinoma Huh7 and cytotoxicity to ileocecal colorectal adenocarcinoma HCT-8 cell lines. In addition, phytotoxicity assays were conducted and revealed that some extracts inhibited tomato rootlet elongation and seed cress germination. In 2017, Umpiérrez et al., investigating the extracts from *Artemisia absinthium* L. and *Eupatorium buniifolium* and their effects on different seeds and insects, noticed that both extracts affected the tomato seeds' relative germination, germination rates and the length that roots reach when exposed to high doses [129]. When acute toxicity test was conducted to honeybees LD_{50} values were higher than those US EPA (2012) indicates, meaning both extracts were considered safe [168]. Furthermore, exposure of 3% (v/v) of *Eupatorium buniifolium* extract to Cetia variety led to acute toxic effects on whiteflies. On the other hand, 4.5% (v/v) led to necrotic effects on the vegetative parts of the plant. Cell cultures, *Caenorhabditis elegans* and hen's eggs were exposed to rosemary, *Citrus* and *Eucalyptus* oils by Lanzerstorfer et al., [169]. A dose dependent decrease of cell viability with IC_{50} ranging between 0.08 to 0.17% (v/v) was observed. *Caenorhabditis elegans* mean LC_{50} value for all oils was 0.42% (v/v). Moreover, the oils led to mucous membrane irritation signs.

Based on the available literature data and the legislation on biopesticides, the importance of evaluation of potential hazards that plant extract and essential oils might oppose to nontarget plants, insects, etc. is highlighted. Although in most cases the toxic effects are dose-dependent, occasionally even at low concentrations they can cause adverse effects. Especially for plant extracts the potential toxic effects of the solvent used as carrier should also be considered.

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Table 6. Recent studies on toxicity assessments of plant extracts/essential oils.

Extract	Assay	Method	References
Prasium majus L., *Glaucium flavum* Crantz, *Daucus lopadusanus* Tineo, *Periploca angustifolia* Labill, *Echinops spinosissimus* Turra, *Hypericum aegypticum* L.	*Solanum lycopersicum*		[78]
Prasium majus L., *Glaucium flavum* Crantz, *Daucus lopadusanus* Tineo, *Periploca angustifolia* Labill, *Echinops spinosissimus* Turra, *Hypericum aegypticum* L.	Hepatocarcinoma Huh7 cell lines/ Ideocecal colorectal adenocarcinoma HCT-8 cell lines	MTT-based colorimetric assay	
Artemisia absinthium L.	*Solanum lycopersicum* L. (Mirella and Cetia seeds)		[129]
	Apis mellifera L.	EPA OCSPP 850.3020 and complete exposure test	
Eupatorium buniifolium	*Solanum lycopersicum* L. (Mirella and Cetia seeds)		
	Apis mellifera L.	EPA OCSPP 850.3020 and complete exposure test	
	Solanum lycopersicum L. (Cetia seeds) and whitflies	Greenhouse assay	
Rosemary oil, Citrus oil, Eucalyptus oil	HeLa cell lines/ Caco-2 cell lines/ STF1 cell lines	Resazurin-based *in vitro* toxicology assay	[169]
	Caenorhabditis elegans		
	Hen's eggs (Lohmann classic brown chicken)		

6. Conclusions and Future Perspectives

Botanical pesticides have long been touted as attractive alternatives to synthetic chemical pesticides for pest management as they reputedly pose little threat to the environment and to human health. They are assumed to be harmless for the farmers, easily biodegradable and less toxic to non-target organisms. The growing number of studies related to the herbicidal, insecticidal, and antimicrobial effects of plant extracts demonstrate their effectiveness and suitability as sustainable and environment-friendly biopesticides. Their various and novel modes of action are attributed to

the specific phytochemical compositions, which are affected by several factors such as plant species or cultivar, geographical origin, environmental conditions, and agricultural practices. In addition, the choice of the extraction method was found to be of primary importance for the quantity and quality of the phytochemicals. In general, "green methods" are suggested considering the potential impact in the environment. More environmentally friendly methods must be adopted and developed focusing on less hazardous solvents as well as reduction of energy consumption and safety, in terms of circular economy.

Considering the increase of population and simultaneously the increasing demand for food, the use of biopesticides is an ecological solution to crop protection. Nevertheless, measures should be taken in order that the cultivation of the raw material (plants) for the production of biopesticides will not affect the global nutritional sufficiency and will not press food production. Moreover, agricultural waste as source of active compounds could be a promising, circular, and cheap raw material for biopesticides.

A significant challenge for biopesticides development is the increase of their effectiveness. A reason for restricted use of biopesticides by the farmers is due to the high degradation rate owing to their volatility, which leads to multiple treatments and increased production cost. To ensure their effectiveness and stability, the formulation of the biopesticides must be improved with minimum influence of external environmental factors, such as temperature. Nanotechnology is a promising science in recent times, with a huge potential to provide novel approaches and solutions in the biopesticide sector, enhancing the stability and efficiency of biopesticide nano-formulations. This means that it is necessary to intensify the biopesticides development and researchers must focus on their production, formulation, and application.

However, several challenges need to be addressed before their commercialization. Biopesticides and related products should be evaluated in a more biological, ecological, and economic context. Up to now, most bioassays were conducted at laboratory scale. However, the few data of experiments in the field significantly limit the commercialization of the biopesticide product. It needs further investigation to reassure the effectiveness of the biopesticide in real conditions, developing suitable formulations which protect the compounds and release them slowly to the environment.

A key factor to determine the suitability of biopesticides is the regulatory approval. In general, there is a strict framework for authorization which delays the promotion of the product. As it concerns low-risk and eco-friendly products, the biopesticides must not be evaluated in the same way as the chemical pesticides. Thus, the approval of the application file of a biopesticide by the authorities should be a simple, rapid, less expensive procedure, different from the chemical pesticides to facilitate the registration of the biopesticide products.

Farmers and society at large should benefit from the use of biopesticides. Regarding farmers, the effectiveness and reliability of biopesticides compared to synthetic chemical pesticides are the most important criteria for their acceptability. Emphasis should be placed on the benefits of the biopesticides' use. This could be supported by public-funded programs, as well as pesticides firms in order to inform the farmers about availability, use and advantages for adopting biopesticides. This is in line with Farm to Fork Strategy which aims to ensure food safety in an environmentally sustainable manner maximizing simultaneously environmental, health and social benefits.

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